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(54) Title: METHOD OF POLYPEPTIDE RENATURATION

(57) Abstract: The present invention relates to a method for producing a polypeptide comprising a Duffy binding like (DBL) domain, wherein the method comprises: (i) expressing the polypeptide in a bacterium or as a non-secreted polypeptide in yeast; (ii) extracting the expressed polypeptide from the bacterium or yeast and denaturing the polypeptide; (iii) refolding the extracted polypeptide in the presence of arginine and urea; and optionally (iv) recovering the refolded polypeptide.

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METHOD OF POLYPEPTIDE RENATURATION

<u>Field of the Invention</u>

     The present invention relates to a method of producing malaria polypeptides

5   in a bacterium or yeast.

<u>Background of the invention</u>

     Malaria is a major public health problem in many parts of the tropical world

with millions of deaths occurring annually from this disease. Various species of

10   *Plasmodium* are responsible for malaria including *Plasmodium falciparum* and

Plasmodium vivax and as drug resistant strains of these pathogens have emerged

there is an urgent need for the development of vaccines that can provide effective

protection against them.

     One potential target for vaccines against malaria is to prevent the entry of the

15   parasite into the erythrocyte. The invasion of erythrocytes by the parasite is mediated

by specific interactions between receptors on the erythrocyte and ligands on the

parasite (C. E. Chitnis *et al*, 1999. In Malaria: Molecular and Clinical Aspects. Ed.

P. Perlmann and M. Wahlgren. Harwood Academic Publishers. p249-285). The

ligands on the parasite responsible for this interaction belong to a family of

20   erythrocyte binding proteins known as the EBP family and these include the Duffy

binding protein of *Plasmodium vivax* (PvDBP) and the 175kd erythrocyte binding

antigen of *Plasmodium falciparum* (EBA-175).

     The members of the EBP family share similar structural features. The

extracellular domains of EBP family members contain two conserved cysteine rich

25   regions, regions II and VI, at their amino and carboxyl ends respectively. The

functional receptor-binding domain of PvDBP has been mapped to region II (Chitnis

C. E. and Miller L. H. , 1994, J. Ex. Med. **180**, p497-506.). In EBA-175, which

contains a tandem duplication (F1 and F2) of the N-terminal cysteine- rich region,

the functional binding domain maps to the F2 region (Sim B. K. L. *et al*, 1994

30   *Science*. **264**, p1941-1944). The binding domains of the PvDBP and EBA-175

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proteins are therefore known as PvRII and PfF2 respectively. PvRII binds the Duffy antigen on human erythrocytes whilst PfF2 binds sialic acid residues of glycophorin A on human erythrocytes.

The binding domains PvRII and PfF2 belong to a family of conserved

5   cysteine-rich domains that are referred to as Duffy-binding-like (DBL) domains because the first binding domain to be identified was derived from the *Plasmodium vivax* Duffy binding protein. Other members of the DBL family include the cysteine-rich domains found in the extracellular region of the PfEMP-1 family of proteins from *Plasmodium falciparum* (Baruch *et al*, 1995, *Cell*. **82**, p77-88; Smith *et al*,

10   1995, *Cell*. **82**, 101-110; and Su *et al*, 1995. *Cell*. **82**, p89-100). Some members of the PfEMP-1 family bind specific endothelial receptors such as ICAM-1, chondroitan sulfate A (CSA), hyaluronic acid (HA) and CD31 to mediate cytoadherence of *Plasmodium falciparum*-infected trophozoites and schizonts to the vascular endothelium (C. E. Chitnis *et al*, 1999. In Malaria: Molecular and Clinical Aspects.

15   Ed. P. Perlmann and M. Wahlgren. Harwood Academic Publishers. p249-285). Binding to ICAM-1 in brain capillaries is implicated in the pathology of cerebral malaria and binding to CSA and HA is implicated in sequestration in the placenta, which may lead to complications in pregnancy (Turner *et al*, 1994, *Am. J. Pathol.* **145**, p1057-1071; Fried *et al*, 1996. *Science* **272**, p1502-1504; and Beeson *et al*,

20   2000. *Nature Medicine* **6**, p86-90). Functional DBL domains from PfEMP-1 that mediate binding to ICAM-1 and CSA have been identified and are being developed as vaccines with the goal of eliciting cytoadherence-blocking antibodies (Smith *et al*, 2000, *PNAS*, **97**, p1766-1771; and Buffet *et al*, 1999 *PNAS*, **96**, p12743-12748).

The binding domains of EBP family members and in particular PvRII and

25   PfF2 are promising candidates for vaccines against *Plasmodium vivax* and *Plasmodium falciparum*. Antibodies capable of binding PfF2 block invasion of erythrocytes by *Plasmodium falciparum* and may provide protection against *Plasmodium falciparum* (Narum *et al*, 2000, *Infection and Immunity*, **68**, p1964-1966). However, in order for the binding domains to elicit protective antibodies

30   which will block erythrocyte binding and invasion it is necessary for them to be in

their native conformations.

Although the PfF2 domain has been expressed in baculovirus and the expressed protein was thought to adopt its native conformation the proteins expressed were glycosylated. Malaria parasite proteins are not normally

5   glycosylated. Glycosylation may mask essential epitopes of the protein. This masking may prevent vaccines comprising the recombinant proteins from raising a protective immune response against the malarial parasite. Furthermore, although DBL domain containing proteins can be expressed in baculovirus and mammalian systems it is expensive to do so and yields of protein are relatively poor.

10 The expression of PvRII and PfF2 in bacteria, or as a non secreted polypeptide in yeast, produces recombinant proteins which are not glycosylated and production in bacteria or yeast is less expensive than expression in baculovirus or mammalian systems. However, expression of proteins containing disulfide bridges, which DBL domains contain, in bacteria or as non-secreted proteins in yeast, often

15 results in the accumulation of incorrectly folded biologically inactive protein in insoluble inclusion bodies and expression of the PfF2 and PvRII domains leads to the information of inclusion bodies. This incorrectly folded protein is not suitable for use in a vaccine and although methods to renature proteins recovered from inclusion bodies are known none have been developed for refolding of DBL domains.

20

Summary of the Invention

The present invention provides a method for producing a polypeptide comprising a Duffy binding like (DBL) domain in bacteria or yeast. When proteins comprising DBL domains are expressed in bacteria they accumulate in incorrectly

25 folded, biologically inactive forms in insoluble inclusion bodies. When proteins containing DBL domains are expressed in yeast as non-secreted polypeptides they also form inclusion bodies. The incorrectly folded polypeptide is not suitable for use in vaccines or for other purposes where it is necessary that the polypeptide adopts a biologically active conformation. The present inventors have shown, for the first

30 time, how the accumulated DBL domain containing recombinant polypeptide can be

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recovered from the inclusion bodies and refolded into a biologically active form.

The inventors have shown that, after recovery of the recombinant polypeptide from the inclusion bodies and denaturation, the polypeptides can be refolded by rapid dilution in the presence of urea and arginine so that they adopt a biologically active

5   conformation. The inventors have further demonstrated that after refolding, if the arginine is removed prior to the removal of the urea that the yield of refolded polypeptide achieved is maximised.

One advantage of the method of the present invention is the polypeptides obtained are non-glycosylated. DBL domain containing polypeptides are not

10   naturally glycosylated. Although methods for obtaining biologically active DBL domain containing polypeptides are known in the art these use baculovirus or mammalian cell systems to express the polypeptide and the recombinant polypeptides obtained are glycosylated. Glycosylation may mask important epitopes of the polypeptide and prevent vaccines containing these polypeptides from raising a

15   protective immune response. A further advantage of the present method over those systems using baculovirus or mammalian cells for expression is that it is far less expensive.

Accordingly the present invention provides a method of producing a cysteine rich polypeptide. In particular, the present invention provides a method of producing

20   a polypeptide comprising a Duffy binding like (DBL) domain, wherein the method comprises:

        (i)   expressing the polypeptide in a bacterium, or as a non-secreted polypeptide in a yeast;

        (ii)   extracting the polypeptide from the bacterium or yeast and denaturing

25   the polypeptide;

        (iii)   refolding the extracted polypeptide in the presence of arginine and urea; and optionally

        (iv)   recovering the refolded polypeptide.

The present invention further provides

30   -   a pharmaceutical composition obtainable or obtained by a method of

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the invention; and

-       a vaccine composition obtainable or obtained by a method of the
        invention.

The invention also provides a method for the identification of a substance that

5   modulates the interaction between a polypeptide produced by a method of the
invention and a host cell receptor involved in the entry of a parasite into a host cell,
wherein the method comprises:

(i)   contacting the receptor with the polypeptide in the
        presence of a test substance;

10   (ii)   determining the effect of the test substance on the
        interaction between the receptor and polypeptide
        thereby to determine whether the test substance is
        capable of modulating the interaction between the
        receptor and polypeptide.

15   The invention further provides a method of treating or preventing malaria in
an individual comprising immunising the individual with a pharmaceutical
composition or a vaccine of the invention.

Brief Description of the Figures

20   **Figure 1. Purification and refolding of recombinant PvRII expressed in
E. coli. A. Coomasie stained SDS-PAGE gel.** Molecular weight standards (MW);
whole cell extract (WC); inclusion bodies (IB); elute fractions after purification by
metal affinity chromatography (Ni), ion-exchange chromatography (IEX) and gel
permeation chromatography (GPC). **B. Western blot.** Rabbit antiserum raised

25   against a 43 amino acid peptide derived from PvRII was used to detect PvRII.
Molecular weight standards (MW); whole cell extract (WC); inclusion bodies (IB);
elute fractions after purification by metal affinity chromatography (Ni), ion-exchange
chromatography (IEX) and gel permeation chromatography (GPC).

**Figure 2. Purification and refolding of recombinant PfF2 expressed in *E.

30   coli*. A. Coomasie stained SDS-PAGE gel.** Molecular weight standards (MW);

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whole cell extract (WC); inclusion bodies (IB); elute fractions after purification by metal affinity chromatography (Ni), ion-exchange chromatography (IEX) and gel permeation chromatography (GPC). **B. Western blot.** A mouse monoclonal antibody raised against 5-His was used to detect PfF2, which contains a 6-His fusion

5   at the C-terminus.

Figure 3. Silver-stained SDS-PAGE gels of refolded, purified PvRII (A) and PfF2 (B). Different amounts (0.6 μg, 1 μg, 2 μg) of refolded and purified recombinant PvRII and PfF2 were separated by SDS-PAGE and detected by silver staining to determine levels of purity.

10   **Figure 4. Mobility of refolded PvRII (A) and PfF2 (B) before and after reduction.** Refolded PvRII and PfF2 have slower mobility by SDS-PAGE after reduction with dithiothreitol (+DTT) indicating the presence of disulfide linkages.

Figure 5. RP-HPLC profile of refolded, purified PvRII (A) and PfF2 (B). Refolded, purified PvRII and PfF2 were analyzed by reverse phase chromatography

15   on a C8 column. Both elute as single, symmetric peaks indicating that they contain a single, homogeneous population of conformers.

Figure 6. Gel filtration chromatography profile of refolded and purified PvRII (A) and PfF2 (B). Both PvRII and PfF2 elute as single peaks with the expected mobility on a Superdex 75 gel filtration column (Pharmacia). Elution times

20   of protein standards of known size (1, bovine serum albumin 66 kD; 2, carbonic anhydrase 29 kD; 3, cytochrome C 12.4 kD; 4, aprotinin 6.5 kD) are shown.

Figure 7. CD spectra of refolded PvRII and PfF2. A. CD spectra of denatured and refolded PvRII. B. CD spectra of refolded PvRII and PfF2.

Figure 8. Erythrocyte binding assay with refolded and purified PvRII

25   **(A) and PfF2 (B). A. Binding specificity of refolded PvRII.** Refolded PvRII (RF) binds human Duffy positive (Fya+b+) but not human Duffy negative (Fya-b-) erythrocytes. **B. Binding specificity of refolded PfF2.** Refolded PfF2 (RF) binds human (Hu) and rhesus (Rh) erythrocytes but not neuramindase-treated (Neu) human and rhesus erythrocytes.

30   **Figure 9. Reactivity of rabbit antisera raised against refolded, purified**

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PvRII and PfF2 as determined by ELISA. A. Rabbit antisera raised against PvRII. Reactivity of rabbit antisera raised against refolded PvRII as determined by ELISA. B. Rabbit antisera raised against PfF2. Reactivity of rabbit antisera raised against refolded PfF2 as determined by ELISA.

5   **Figure 10. Detection of inactive PvRII by immunoprecipitation and Western blot following pre-absorption with Duffy positive erythrocytes.** Refolded PvRII was immunoprecipitated, separated by SDS-PAGE and detected by Western blot either without pre-absorption (-) or after pre-absorption with Duffy positive (D+) or Duffy negative (D-) human erthrocytes.

10

Brief Description of the Sequences

          SEQ ID NO: 1 shows the amino acid sequence of the PvRII domain of the PvDBP protein of *Plasmodium vivax.*

          SEQ ID NO: 2 shows the amino acid sequence of the PfF1 domain of the
15   EBA-175 of *Plasmodium falciparum.*

          SEQ ID NO: 3 shows the amino acid sequence of the PfF2 domain of the EBA-175 of *Plasmodium falciparum.*

          SEQ ID NOs: 4 and 5 show the nucleotide sequences of the primers used to amplify the PvRVII domain of PvDBP in the Example.

20   SEQ ID NOs: 5 and 6 show the nucleotide sequences of the primers used to amplify the PfF2 domain of EBA-175 in the Example. SEQ ID NO: 7 is a further primer sequence.

Detailed description of the Invention

25   *DBL domain containing polypeptides*

          The present invention provides a method for producing a polypeptide comprising a DBL domain. Typically DBL domains are conserved, cysteine-rich domains that share significant homology with region II, the erythrocyte binding domain of the *Plasmodium vivax* Duffy binding protein. Region II of PvDBP

30   (PvRII) contains 12 cysteines. DBL domains from other erythrocyte binding proteins

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such as EBA-175 usually have these 12 conserved cysteines and in addition may
have others not present in PvRII. DBL domains from PfEMP-1 usually contain the
first 10 cysteines of PvRII and in addition may contain others. Besides the conserved
cysteines other short conserved amino acid stretches containing hydrophobic amino

5   acid residues may be shared by DBL domains. Sequence homology between different
DBL domains is typically in the range of 20-90%.

        Preferably the polypeptide will be from a pathogen and in particular from a
parasite. In a preferred embodiment of the invention the parasite will be one which is
responsible for malaria. The polypeptide may be derived from a drug resistant strain

10   of a particular pathogenic or parasitic species. In a particularly preferred embodiment
of the invention the polypeptide will be one from *Plasmodium falciparum* or
Plasmodium vivax.

        In a preferred embodiment of the invention the polypeptide will be a parasite
protein involved in the binding of the parasite to host cells and in particular to host

15   erythrocytes or vascular endothelium. Examples of polypeptides which may be used
include the binding domains (DBL domains) of the PvDBP protein of *Plasmodium
vivax*, the EBA-175 protein and the PfEMP-1 family of proteins of *Plasmodium
falciparum*. The polypeptide may be a homologue of any of these proteins and in
particular a homologue of EBA-175 encoded by *Plasmodium falciparum*. Examples

20   of preferred PfEMP-1 family proteins are those responsible for binding to vascular
and in particular brain, placental or foetal vascular endothelium and these include
those responsible for binding to ICAM-1, chondroitan sulfate A (CSA), hyaluronic
acid (HA) and CD31. Also included are homologs of these proteins from other
pathogenic species and in particular from other species of parasites which include a

25   DBL domain and are responsible for binding of the parasite or pathogen to host cells.

        The DBL domain containing polypeptide may be a fragment or a variant of a
parasitic polypeptide and in particular of those parasite polypeptides responsible for
binding to a host cell. Preferably the fragment or variant will be capable of
specifically binding to the host cell or host cell receptor for the parasite. Particularly

30   preferred fragments include those of the above mention proteins and in particular the

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PvRII domain of PvDBP, the PfF2 domain of EBA-175 and a domain from PfEMP-1 family protein responsible for receptor binding. Also included are portions of these domains containing a DBL domain and preferably capable of binding a host cell receptor.

5   In a preferred embodiment of the invention the polypeptide will have the amino acid sequence of SEQ ID NOs: 1 to 3 or be a fragment or variant of these sequences. Preferably the fragment or variant will be able to bind the Duffy antigen (for the variants and fragments of SEQ ID NO: 1) or Glycophorin (for the variants or fragments of SEQ ID NOs: 2 and 3). Typically the polypeptide will comprise from 4

10   to 14 cysteine residues, preferably 8 to 12 cysteine residues and more preferably 10, 11 or 12 cysteine residues.

The term "variant" refers to a polypeptide which has the same essential character or basic biological functionality as a DBL domain. Typically the variant can bind a host cell receptor, preferably a receptor for a parasite polypeptide, more

15   preferably a host cell receptor for a malaria polypeptide and in particular a host cell receptor for a *plasmodium falciparum* or *plasmodium vivax* polypeptide. In an especially preferred embodiment of the invention the variant can bind the Duffy antigen or glycophorin.

Amino acid substitutions may be made, for example from 1, 2 or 3 to 10, 20

20   or 30 substitutions. Conservative substitutions may be made, for example according to Table I. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other.

Table I

25

ALIPHATIC	Non-polar	G A P
		I L V
	Polar-uncharged	C S T M
		N Q
	Polar-charged	D E
		K R
AROMATIC		H F W Y

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Shorter polypeptide sequences are within the scope of the invention and particular fragments of the above mentioned polypeptides. For example, a peptide of at least 20 amino acids or up to 50, 60, 70, 80, 100, 150 or 200 amino acids in length is considered to fall within the scope of the invention as long as it is able to bind a

5   host cell receptor which binds a parasite polypeptide. In particular, but not exclusively, this aspect of the invention encompasses the situation when the protein is a fragment of the complete protein sequence of PvDBP, EBA-175, homologues of the EBA-175 protein and PfEMP-1 family polypeptides

Typically, polypeptides with more than about 20% identity preferably at least

10   30% or at least 50% and particularly preferably at least 70% at least 90% or at least 99% identity, with the amino acid sequences of SEQ ID NOs: 1 or 3, or of the DBL domains contained therein are considered as variants of the polypeptides within the meaning of the invention. Such variants may include allelic variants and the deletion, modification or addition of single amino acids or groups of amino acids within the

15   protein sequence, as long as the peptide maintains a basic biological functionality of the DBL domain.

The invention also includes homologues of the above mentioned polypeptides and in particular those from parasitic or pathogenic species, preferably those from malaria parasites and in particular those from *plasmodium vixax* and *plasmodium*

20   *falciparum*. Homologues will preferably be able to bind a host cell receptor for a parasite/pathogen, preferably a receptor involved in binding a malaria parasite and in particular a receptor involved in binding a *plasmodium vixax* or *plasmodium falciparum* polypeptide.

Software for performing BLAST analyses and identifying homologues is

25   publicly available through the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pair (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the

30   neighbourhood word score threshold (Altschul *et al*, 1990). These initial

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neighbourhood word hits act as seeds for initiating searches to find HSPs containing

them. The word hits are extended in both directions along each sequence for as far as

the cumulative alignment score can be increased. Extensions for the word hits in

each direction are halted when: the cumulative alignment score falls off by the

5   quantity X from its maximum achieved value; the cumulative score goes to zero or

below, due to the accumulation of one or more negative-scoring residue alignments;

or the end of either sequence is reached. The BLAST algorithm parameters W, T and

X determine the sensitivity and speed of the alignment. The BLAST program uses as

defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff and

10   Henikoff (1992) *Proc. Natl. Acad. Sci.* USA 89: 10915-10919) alignments (B) of 50,

expectation (E) of 10, M=5, N=4, and a comparison of both strands.

The BLAST algorithm performs a statistical analysis of the similarity

between two sequences; see e.g., Karlin and Altschul (1993) *Proc. Natl. Acad. Sci.*

USA 90: 5873-5787. One measure of similarity provided by the BLAST algorithm

15   is the smallest sum probability (P(N)), which provides an indication of the

probability by which a match between two nucleotide or amino acid sequences would

occur by chance. For example, a sequence is considered similar to another sequence

if the smallest sum probability in comparison of the first sequence to the second

sequence is less than about 1, preferably less than about 0.1, more preferably less

20   than about 0.01, and most preferably less than about 0.001.

Any combination of the above mentioned degrees of sequence identity and

minimum sizes may be used to define polypeptides of the invention, with the more

stringent combinations (i.e. higher sequence identity over longer lengths) being

preferred.

25   The DBL domain containing polypeptides may contain heterologous regions.

For example the polypeptides may contain members of a specific binding pair to

allow their purification. Typically this will be a purification tag which as used herein

is an amino acid sequence that can bind to a target under suitable conditions.

Usually the target will be provided by a manufacturer, who will recommend a

30   purification tag and suitable conditions. The member of the specific binding pair may

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be present anywhere in the polypeptide but is preferably present at the N or C
terminus of the polypeptide and may be removed by cleavage.

In a preferred embodiment, the purification tag is a His-Tag. Typically the
His-tag comprises multiple contiguous histidine residues, preferably from 3 to 20,
5   more preferably from 4 to 10 most preferably 6. Typically the His-tag will be
positioned at either or both of the N- and C- terminals of the fusion polypeptide. The
preferred target for the His-tag is NiNTA, which is available commercially, e.g. from
Qiagen (Germany). Other preferred purification tags include a self-cleaving chitin-
based ligand system, a sequence capable of binding maltose, a nickel-based ligand
10   system and a glutathione S-transferase based system.

If the polypeptide is for use in an assay it may be a fusion polypeptide with
an enzyme, transcription factor domain, green fluorescent protein or other suitable
reporter sequence.

The polypeptide may also comprises a cleavage site allowing release of other
15   regions of the polypeptide from the DBL domain for example the purification tag. A
cleavage site is any amino acid sequence that facilitates cleavage of fusion
polypeptide to release the polypeptide of interest.

Polypeptide expression
20   Typically, the nucleotide sequences encoding the DBL domain containing
polypeptide, fragment or variant will be inserted into a vector, introduced into a
suitable host and expressed therein. Typically a prokaryotic bacterium, and in
particular a gram negative bacterium, will be used as the host cell. In an especially
preferred embodiment of the invention the bacterium used will be *E.coli*. Alternative
25   prokaryotic bacteria and expressions may be used such as, for example *Lactobacillus*
or *Bacillus subtilis*. In one embodiment of the invention the polypeptide may be
expressed as a non-secreted protein in yeast strains such as *Saccharomyces cerivisae*
or *Pichia pastoris*. This is because in yeast, proteins are not glycosylated if they are
not targeted for secretion.

30   Typically the polypeptide will include a purification tag to allow its recovery.

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Suitable cloning methods, vectors, bacteria or yeast strains and methods for expression of polypeptides in bacteria or yeast are well understood by persons skilled in the art, and include techniques such as those disclosed in Sambrook *et al*, Molecular Cloning: a Laboratory Manual, 2nd Edition, CSH Laboratory Press, 1989,

5   the disclosure of which is included herein in its entirety by way of reference. After expression of the recombinant polypeptide typically the polypeptide will accumulate in inclusion bodies and these are then recovered from the bacterium or yeast cell. Typically the recombinant polypeptide is recovered using a method as follows:

-       the bacteria or yeast are harvested, the cells lysed,

10   typically by sonication, and the inclusion bodies
                recovered by centrifugation;

-       the inclusion bodies are solubilised, preferably in
        guanidine-HCl at a concentration of from 4 to 8 Molar
        and in particular at a concentration of 6 Molar;

15   -   insoluble debris are removed; and

-       the recombinant polypeptide is recovered and retained
        in denaturing conditions before refolding.

In a preferred embodiment the buffer for the solubilisation of inclusion bodies contains 10mM Tris HCl, pH 8.0, 100mM NaH_2Po_4, 100mM NaCl , 6M

20   guanidine–HCl and 10mM DTT. Typically the solubilisation is carried out from 5 to 24 hours, preferably 8 to 12 hours and typically with constant stirring. Preferably the solubilisation is carried out at a temperature from 15 to 25°C, more preferably from 20 to 25°C and in particular at 23 °C.

If the polypeptide is His tagged it may be recovered using metal affinity

25   chromatography and typically a Ni-NTA column.

Refolding of the polypeptide

After the polypeptide is recovered from the inclusion bodies it is typically refolded by dilution in the presence of arginine and urea. Typically before dilution

30   the polypeptide is at a concentration of at least 1 mg/ml and preferably at a

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concentration of at least 4mg/ ml. The solution containing the polypeptide is then diluted using a solution containing arginine and urea. Preferably the dilution is a rapid dilution typically performed by adding the solution containing the polypeptide to the other solution rather than *vice versa*.

5   Typically the polypeptide is diluted with the solution by a factor of 50 to150 fold, preferably from 75 to125 fold and more preferably between 90 and 100 fold. In a particularly preferred embodiment of the invention the dilution factor used is 100 fold. Typically, the polypeptide is diluted to a concentration of from 10 to 100μg/ml, preferably to a concentration of from 30 to 70 μg/ml and more preferably from 40 to

10   60 μg/ml. In a preferred embodiment of the invention the polypeptide is diluted to a concentration of 45 μg/ml.

Typically the ratio of arginine to urea in the solution used for dilution is from 1:1 to 1:5, preferably from 1:1 to 1:3 and more preferably the ratio is 1:2. Preferably the urea is present at a concentration of from 0.5 to 1.5 M, more preferably from 0.7

15   to 1.2 M and even more preferably from 0.9 to 1.1 M. Preferably, the arginine is present at a concentration of from 0.1 to 1 M, preferably from 0.3 to 0.7 M and more preferably between 0.4 and 0.6 M. In a particularly preferred embodiment of the invention the urea is present at a concentration of 1.0 M and/or the arginine is present at a concentration of 0.5 M.

20   The pH of the solution may be from pH 4.0 to pH 9.0, preferably from pH 4.0 to pH 7.0 and more preferably from pH 4.0 to pH7.0. The pH used will depend on the particular protein and the optimal pH and other conditions may be readily determined by titrating the conditions against the yield of correctly folded protein obtained.

In a preferred embodiment of the invention the rapid dilution solution in addition to

25   arginine and urea also comprises 50mM phosphate buffer - (pH 7.2 for PvRII and pH 5.8 for PfF2), reduced glutathione (GSH) at a concentration from 0.2 mM to 2 mM and oxidised glutathione (GSSG) at a concentration of from 0.02 mM to 0.2 mM. The ratio of reduced glutathione to oxidised glutathione is preferably from 5:1 to 25: 1, more preferably from 8:1 to 15:1 and even more preferably from 10:1 to

30   15:1. In a preferred embodiment of the invention the ratio will be 10:1.

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In an especially preferred embodiment of the invention the refolding is carried out in a buffer comprising 1mM reduced glutathione (GSH), 0.1mM oxidised glutathione (GSSG), 1M urea and 0.5M arginine.

Typically the refolding is carried out at a temperature from 5 to 37°C,
5   preferably at from 5 to 20°C and more preferably from 5 to 15°C. In a particularly preferred embodiment of the invention the refolding is carried out at 10°C. Typically the refolding step is carried out for a period of from 2 to 64 hours, preferably from 12 to 48 hours, more preferably from 24 to 36 hours. In a preferred embodiment of the invention the refolding step is carried out at a temperature of from 5 to 15°C and for a
10   period of from 12 to 48 hours. In a particularly preferred embodiment the refolding step is carried out at a temperature of 10°C and for a period of 36 hours.

Multiple additions of the denatured, extracted polypeptide may be made to the refolding solution. This allows refolding larger amounts of polypeptide without loss of yield of refolded polypeptide. Preferably such multiple additions will be
15   made with intervals of ½ to 3 hours, more preferably 1 to 2 or 1½ to 2½ hours, e.g. approximately 2 hours. It is preferred to make 2, 3, 4 or 5 additions, more preferably 3 additions. Desirably, a final concentration of peptide of 40 to 80µg/ml, e.g. 50 to 70µg/ml, preferably approximately 60µg/ml will be achieved in the refolding solution, either by a single addition of peptide, or preferably by multiple additions as
20   just described. In a preferred embodiment of the invention denatured polypeptide is added three times with intervals of approximately 2 hours between additions to achieve a final concentration of approximately 60µg/ml.

Removal of arginine and urea from the refolded polypeptide
25   After refolding the polypeptide is recovered. The recovery process may involve the removal of the arginine and urea. As the present inventors have demonstrated that removing the arginine prior to the removal of the urea substantially increases the yield of correctly refolded protein the recovery process will more preferably include removal of arginine and then subsequently the removal of the
30   urea.

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The removal of arginine is typically carried out by dialysis. Typically the dialysis is carried out for from 12 to 72 hours, preferably from 24 to 48 hours and in particular it is carried out for 48 hours. The refolding solution is typically dialysed against a dialysis buffer comprising a phosphate buffer and urea. Preferably the

5   concentration of urea in the dialysis buffer is equimolar or approximately equimolar with the concentration of urea in the solution containing the protein. Typically the dialysis buffer will comprise 50mM phosphate buffer at a pH appropriate to the particular protein (pH 6.5 for PvRII and pH 5.8 for PfF2) and urea at an equimolar concentration to the concentration of urea in the solution being dialysed.

10   The urea may be removed by a purification method such as ion exchange chromatography either separately or in combination with gel filtration chromatography. Typically if ion exchange chromatography is used the refolded protein is loaded onto an equilibriated sepharose column and after washing the bound protein eluted with a linear gradient of equilibration buffer containing NaCl

15   Preferably fractions containing the protein of interest are then pooled and purified using gel filtration chromatography.

Assessment of the biological activity of the polypeptides of the invention

Preferably the refolded polypeptides of the invention are in their native

20   conformation or in a conformation similar to their native conformation. Preferably the polypeptide is able to specifically bind its normal ligand on the host cell. It is especially preferable that polypeptides of the invention to be used in the vaccines or assays of the invention adopt their native conformation or a conformation closely approximating their native conformation. Preferably at least the regions of the

25   polypeptide which interacts with and/or bind the host cell receptor will adopt their native conformation.

Assays such as reverse phase chromatography, circular dichroism analysis and crystallography may be used to assess whether the polypeptide has refolded and/or whether the refolded polypeptide obtained is present in a single or variety of

30   conformations. Refolded polypeptides where the majority, and preferably all, of the

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polypeptide is in a single, and preferably the native, conformation are especially preferred for use in the assays, vaccines and methods of the invention.

In a particular preferred embodiment of the invention the binding of the refolded polypeptide to a host cell or the isolated host cell receptor it is specific for
5   will be assessed. Polypeptides which do bind to these are preferred for use in the vaccines or assays of the invention. Typically, in such assays, the binding of the polypeptide to cells, preferably erythrocytes, with the receptor and lacking the receptor will be compared.

Where the polypeptide is EBA-175 or a fragment, variant or homologue
10   thereof the ability of the polypeptide to bind to glycophorin may be assessed. The ability of the polypeptide to bind to neuraminidase treated erythrocytes (which lack sialic acid residues) and untreated human erythrocytes may be used to determine if the polypeptide can specifically bind glycophorin in its normal state and hence normal human erythrocytes.

15   Where the polypeptide is PvDBP or a fragment, variant or homologue thereof the ability of the polypeptide to bind to the Duffy antigen may be determined by comparing the binding of the polypeptide to Duffy positive human erythrocytes and Duffy negative erythrocytes.

The suitability of particular refolded polypeptides for use in the assays and
20   methods of the invention may also be determined by their ability to raise antibodies which block the binding of the native protein they are derived from to its receptor and/or prevent the entry of the parasite into the host cell. This may be done by raising anti-sera against the polypeptide in animals such as rabbits and then assessing whether the anti-sera can inhibit or prevent binding to the host cell receptor or the
25   host cell itself. Polypeptides of the invention which can raise such anti-sera are especially preferred for use in the vaccines, assays and methods of the invention.

Assays using the polypeptides of the invention

The invention provides methods of identifying substances which can bind to a

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polypeptide of the invention and more preferably can bind to the polypeptide and hence prevent the binding of the polypeptide to the host cell receptor for it.

In one embodiment of the invention the binding of the polypeptide to the host cell receptor is compared in the presence and absence of the test substance.

5   The host cell receptor may be in an isolated form or present on a cell. Preferably the receptor is a receptor associated with the entry of a malaria parasite, typically *Plasmodium falciparum* or *Plasmodium vivax*, into the host cell. In a preferred embodiment of the invention the receptor is the Duffy antigen or glycophorin. The cell may either naturally express the receptor or express the

10   receptor as it has been transformed by a nucleic acid encoding the receptor. Preferably the cell is an erythrocyte. The binding of the polypeptide to cells with and without the receptor is typically compared in the presence and absence of the test substance. In cell based assays the actual entry of hte polypeptide inot the cell may be assessed.

15   In such methods the test substance may inhibit the binding of the receptor to the polypeptide either by the candidate substance binding the polypeptide or by the test substance binding the receptor. Thus the method can identify not only substances that bind the polypeptide, but also substances that act as mimics of the polypeptide.

20   Alternatively the binding of the test substance to the polypeptide is determined. Substances that are identified as able to bind the polypeptide may then be assessed to see if they can inhibit the binding of the polypeptide to its receptor. In the assays the polypeptide may be immobilised on a solid support or may be in solution. The binding of the test substance to the polypeptide or the binding of the

25   polypeptide to the receptor may be determined by measuring a characteristic of the polypeptide or receptor that changes upon binding, such as spectroscopic changes. Suitable test substances which can be tested in the above assays include combinatorial libraries, defined chemical entities and compounds, peptide and peptide mimetics, oligonucleotides and natural product libraries, such as display (e.g.

30   phage display libraries) and antibody products.

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Typically, organic molecules will be screened, preferably small organic molecules which have a molecular weight of from 50 to 2500 daltons.

Test substances may be used in an initial screen of, for example, 10 substances per reaction, and the substances of these batches which show inhibition of

5   binding of the polypeptide to the receptor or which bind to the polypeptide tested individually. Test substances may be used at a concentration of from 1nM to 1000μmM, preferably from 1μM to 100μM, more preferably from 1μM to 10μM. Preferably, the activity of a test substance is compared to the activity shown by a known inhibitor of receptor - polypeptide binding. A test substance which acts as an

10   inhibitor may produce a 50% inhibition of activity of the receptor. Alternatively, a test substance which acts as an activator may produce 50% of the maximal activity produced using a known activator.

Use of the polypeptides of the invention to make antibodies

15   Antibodies to the refolded polypeptides produced using the method of the invention can be made by use of the following methods. An antibody to the substance may be produced by raising antibody in a host animal against the whole substance or an antigenic epitope thereof (hereinafter "the immunogen"). Methods of producing monoclonal and polyclonal antibodies are well-known.

20   A method for producing a polyclonal antibody comprises immunising a suitable host animal, for example an experimental animal, with the immunogen and isolating immunoglobulins from the serum. The animal may therefore be inoculated with the immunogen, blood subsequently removed from the animal and the IgG fraction purified.

25   A method for producing a monoclonal antibody comprises immortalising cells which produce the desired antibody. Hybridoma cells may be produced by fusing spleen cells from an inoculated experimental animal with tumour cells (Kohler and Milstein, Nature 256, 495-497, 1975).

An immortalized cell producing the desired antibody may be selected by a

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conventional procedure. The hybridomas may be grown in culture or injected intraperitoneally for formation of ascites fluid or into the blood stream of an allogenic host or immunocompromised host. Human antibody may be prepared by *in vitro* immunisation of human lymphocytes, followed by transformation of the

5   lymphocytes with Epstein-Barr virus.

For the production of both monoclonal and polyclonal antibodies, the experimental animal is suitably a goat, rabbit, rat or mouse. If desired, the immunogen may be administered as a conjugate in which the immunogen is coupled, for example via a side chain of one of the amino acid residues, to a suitable carrier.

10 The carrier molecule is typically a physiologically acceptable carrier. The antibody obtained may be isolated and, if desired, purified.

Composition and, vaccines using the polypeptides of the invention

The polypeptides of the invention may be used in vaccines. Typically the

15 vaccines will be used to prevent malaria. A polypeptide of the invention can therefore be used to prevent infection by *Plasmodium faciparum* or *Plasmodium vivax*. The vaccine may be in the form of a pharmaceutical composition which comprises the polypeptide and a pharmaceutically acceptable carrier or diluent. Suitable carriers and diluents include isotonic saline solutions, for example

20 phosphate-buffered saline. A pharmaceutically acceptable adjuvant such as alum may be present. Typically the administration is (and thus the composition is formulated for) parenteral, intravenous, intramuscular, subcutaneous, transdermal, intradermal, oral, intranasal, intravaginal, or intrarectal administration. Typically the vaccine is given 1, 2, 3 or more separate administrations, each of which is separated

25 by at least 12 hours, 1 day, 2, days, 7 days, 14 days, 1 month or more.

An effective amount of the polypeptide is administered to a host, typically a human being. The dose of polypeptide administered as a vaccine may be determined according to various parameters, especially according to the age, weight and condition of the patient to be treated; the route of administration; and the required

30 regimen. A physician will be able to determine the required route of administration

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and dosage for any particular patient. A suitable dose may however be from 10 µg to
1g, for example from 100 µg to 50mg of the polypeptide. These values may
represent the total amount administered in the complete treatment regimen or may
represent each separate administration in the regimen.

5   Substances identified in the assays of the invention may be used to treat
malaria. Such a substance may be used to treat infection by *Plasmodium falciparum*
or *Plasmodium vivax*. In particular those substances identified which can bind to the
polypeptides of the invention, preferably those that can prevent the binding of the
polypeptide to its receptor and more preferably those which are capable of preventing
10   invasion of the host cell by the parasite, will be used in the treatment of malaria. The
condition of a patient suffering from malaria can thus be improved. The symptoms
of malaria may be ameliorated.

          Substances identified according to the screening methods outlined above may
be formulated with standard pharmaceutically acceptable carriers and/or excipients as
15   is routine in the pharmaceutical art. For example, a suitable substance may be
dissolved in physiological saline or water for injections. The exact nature of a
formulation will depend upon several factors including the particular substance to be
administered and the desired route of administration. Suitable types of formulation
are fully described in Remington's Pharmaceutical Sciences, Mack Publishing
20   Company, Eastern Pennsylvania, 17th Ed. 1985, the disclosure of which is included
herein of its entirety by way of reference.

          The substances may be administered to a host, typically a human being, by
enteral or parenteral routes such as via oral, buccal, anal, pulmonary, intravenous,
intra-arterial, intramuscular, intraperitoneal, topical or other appropriate
25   administration routes. An effective amount of a substance is given to a patient. The
amount that is administered will depend upon a number of factors such as the
substance used; the age, weight and condition of the patient; the route of
administration and the required regimen. A physician will be able to determine the
required route of administration and the dosage for any particular patient. A typical
30   amount administered may be from 10µg to 1g, for example from 100µg to 100mg.

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The following Example illustrates the invention

Example

       Plasmid constructs for expression of PvRII and PfF2 in *E. coli*: DNA

5   encoding PvRII with a C-terminal six His fusion was amplified by PCR using

primers 5'- GCA TGC CAT GGA TCA TAA GAA AAC GAT CT 3' and 5' - CGA

GTG TCG ACT CAG TGA TGG TGA TGG TGA TGT GTC ACA ACT TCC TGA

GT - 3' and a plasmid containing the gene encoding *Plasmodium vivax* Duffy binding

protein as template. The PCR product was restricted with NcoI and SalI and cloned

10   in the vector pET28a+ (Novagen Inc., USA) downstream of the T7 promoter to yield

expression plasmid pVPET1. Similarly, DNA encoding PfF2 with a C-terminal six

His fusion was amplified by PCR using primers 5'TCT AGT CCA TGG AAA AGC

GTG AAC ATA TT3' and 5'ACG AGT GTC GAC TCA GTG ATG GTG ATG

GTG ATG ATC GTC ATC ACG TTC TT3' and a plasmid containing the gene

15   encoding *Plasmodium falciparum* EBA-175 as template. The PCR product was

restricted with NcoI and SalI and cloned in the multiple cloning site of the vector

pET28a+ downstream of the T7 promoter to yield plasmid pFPET1. *E. coli* BL21

(DE3) was transformed with plasmids pVPET1 and pFPET1 to yield the strains

BL21 (DE3) pVPET1 and BL21(DE3) pFPET1, which were used for expression of

20   recombinant PvRII and PfF2 respectively.

       Expression of PvRII and PfF2 in *E. coli*: Luria broth (LB) medium

containing kanamycin (50µg/ml) was inoculated with E. coli BL21 (DE3) pVPET1

·or E. coli BL21 (DE3) pFPET1 and cultured overnight at 37°C. Fresh LB medium

containing kanamycin (25µg/ml) was inoculated with the overnight culture at a

25   dilution of 1:50 and cultured at 37°C to an OD_{600nm} of 0.6-0.8. Expression of

PvRII/PfF2 was induced by adding IPTG (iso propyl-1-thio-b-galactopyranoside) to

the culture at a final concentration of 1mM and allowing the culture to grow for

another 4 hours.

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Isolation of inclusion bodies: Induced *E. coli* cultures were harvested by
centrifugation and the cells were washed twice in chilled wash buffer A (10mM tris
HCl pH 8.0, 10mM EDTA, 100mM NaCl). The washed cell pellet was resuspended
in prechilled lysis buffer (10 mM Tris, pH 8.0, 5 mM Benzamidine-HCl, 2 mM
5   PMSF, 10 mM EDTA, 100 mM NaCl, 200 µg/ml Lysozyme) and the cells were
lysed by sonication. The lysed cells were centrifuged at 4°C, the supernatant was
carefully decanted and the pellet containing inclusion bodies was retained. The
inclusion bodies were washed twice with wash buffer B (10mM Tris-HCl, pH 7.2,
10mm EDTA, 100mM NaCl. 2-4M urea, 2% Triton X100) and once with 1 M NaCl.

10   Purification of recombinant PvRII and PfF2 by metal affinity
chromatography under denaturing conditions: Purified inclusion bodies were
dissolved in solublization buffer (10mM Tris HCl, pH 8.0, 100mM NaH_2PO_4, 100mM
NaCl , 6M Guanidine–HCl, 10mM DTT) by stirring at 23°C overnight. Insoluble
debris was removed by centrifugation and the supernatant was retained.

15   Dithiothreitol was removed from the supernatant by filtration using a 10K cut off
Amicon filter and Ni-NTA binding buffer (10mM Tris, pH 8.0, 100mM NaH_2PO_4,
6M Guanidine HCl). The solubilised inclusion bodies were loaded on a Ni-NTA
column that had been equilibrated with equilibration buffer (10mM Tris pH 8.0,
100mM NaH_2PO_4, 6M guanidine-HCl). The column was washed with column wash
20   buffer (10mM Tris pH 6.3, 100mM NaH_2PO_4, 6M guanidine-HCl) and the bound
protein was eluted with elution buffer (10mM Tris pH 4.3, 100mM NaH_2PO_4, 6M
guanidine-HCl). The final concentration of the purified protein was adjusted to
around 4.5 mg/ml with elution buffer.

Refolding PvRII and PfF2 by rapid dilution: Ni-NTA purified PvRII and
25   PfF2 were refolded by 100 fold rapid dilution in refolding buffer (50mM phosphate
buffer - pH 7.2 for PvRII and pH 5.8 for PfF2, 1mM reduced glutathione (GSH),
0.1mM oxidised glutathione (GSSG), 1M urea, 0.5M arginine) to a final protein
concentration of around 45 µg/ml. Refolding was allowed to proceed at 10°C for 36
hours with stirring. At the end of 36 hours, the refolding solution was dialyzed for

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48 hours against dialysis buffer (50mM phosphate buffer - pH 6.5 for PvRII and pH 5.8 for PfF2, 1M urea) to remove arginine before proceeding with purification by ion-exchange chromatography.

The following ranges were tried:

5   Final protein concentration upon dilution: 10 μg/ml to 50 μg/ml

Arginine: 0.2M to 1M

Urea: 0.5M to 3M

Temp: 2-15°C

pH: 4.0 – 8.5

10   **Purification by ion-exchange chromatography and gel filtration chromatography:** Following removal of arginine by dialysis, the refolded protein was loaded on a SP-Sepharose column equilibrated with equilibration buffer (50mM phosphate buffer - pH 6.5 for PvRII and pH 5.8 for PfF2, 1M urea). The column was washed with equilibration buffer containing 100mM NaCl, 1 M urea. The bound

15   protein is eluted with a linear gradient of equilibration buffer containing NaCl (100mM NaCl to 1.5 M NaCl). Fractions containing protein of interest were pooled and purified by gel filtration chromatography using a Superdex 75 column (Pharmacia, Sweden). During gel filtration chromatography 50 mM phosphate buffer (pH 7.2 for PvRII and pH 6.0 for PfF2) containing 200 mM NaCl were used.

20   **Analysis of refolded, purified PvRII and PfF2 by reverse phase chromatography:** Refolded PvRII and PfF2 were loaded on a reverse phase C8 column. The gradient used for elution was developed using Buffer A (0.05% TFA in water) and Buffer B (0.05% TFA in 90% acetonitrile, 10% water. The column was initially equilibrated with 90% Buffer A and 10% Buffer B and reached a

25   composition of 10% Buffer A and 90% Buffer B in 40 minutes.

Erythrocyte binding assay: Refolded purified PvRII and PfF2 were added to normal or enzyme-treated RBCs and the reaction mix was incubated with rocking at room temperature for 1 hour. The reaction mixture was layered over oil and centrifuged to separate the RBCs with bound protein. Bound proteins were eluted

30   from the RBCs with salt (300 mM NaCl) separated by SDS-PAGE and detected by

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Western blot. Rabbit antisera raised against a peptide derived from PvRII and a mouse monoclonal antibody directed against the His tag were used to detect PvRII and PfF2 respectively.

Rabbit antisera raised against refolded PvRII and PfF2 and assays for

5   **inhibition of RBC binding:** COS cells were transfected with a plasmid construct, pHVDR22, designed to express PvRII on the surface of mammalian cells as described earlier (5). Binding of RBCs to transfected COS cells expressing PvRII on the surface was tested using an erythrocyte-binding assay as described earlier (5). Erythrocyte binding assays were performed in the presence of different dilutions of

10   rabbit antisera raised against refolded PvRII. The number of rosettes of COS cells covered with RBCs was scored in 50 fields at a magnification of 40.

Mobility by gel electrophoresis and gel filtration chromatography: Refolded PvRII and PfF2 had the expected mobility on SDS-PAGE gels (38 kD and 42 kD respectively) and were greater than 98% pure as determined by silver staining

15   of SDS-PAGE gels (Figures 1, 2, 3). Recombinant PvRII was recognized in a Western blot by an antibody raised against a peptide derived from PvRII (Figure 1b). Recombinant PfF2 was recognized in a Western blot by an antibody directed against 5X-HIS fused to the C-terminal end of PfF2 (Figure 2b). The mobility of refolded PvRII and PfF2 was determined by SDS-PAGE before and after reduction with

20   dithiothreitol (DTT) (Figure 4). Reduced PvRII and PfF2 had slower mobility on SDS-PAGE gels compared to non-reduced PvRII and PfF2 indicating the presence of disulfide linkages in the refolded proteins (Figure 4). The mobility of refolded PvRII and PfF2 was determined by gel filtration chromatography using a Superdex 75 (Pharmacia) column (Figure 5). Both PvRII and PfF2 had the expected mobility by

25   gel filtration chromatography. No aggregates or oligomers were detected.

Analysis of refolded PvRII and PfF2 by reverse phase chromatography: Reverse phase chromatography can separate proteins based on differences in the hydrophobicity of exposed surfaces. Reverse phase chromatography (RP-HPLC) was used to determine if refolded PvRII and PfF2 contain heterogeneous populations with

30   different conformers or a homogeneous population of native conformers. Both,

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refolded PvRII andPfF2, elute as single, symmetric peaks on C-8 columns by reverse-phase chromatography indicating that they contain a single, homogeneous population of conformers (Figure 6).

5   **CD spectra:** The CD spectra of refolded and denatured PvRII were determined (Figure 7). As expected denatured PvRII did not show presence of any secondary structure. The negative peaks in the near UV range in the CD spectrum of refolded PvRII and PfF2 indicate presence of significant α helical structure. Refolded PvRII and PfF2 had similar CD spectra indicating that they have similar structures.

Erythrocyte binding assay: Refolded PvRII and PfF2 were tested for
10   functional activity in erythrocyte binding assays (Figure 8). Refolded PvRII binds Duffy positive human RBCs but not Duffy negative human RBCs. Refolded PfF2 binds normal human and rhesus monkey RBCs but not neuraminidase-treated human or rhesus RBCs that lack sialic acid residues. Refolded PvRII and PfF2 thus bind RBCs with the correct specificity indicating that they are folded in their native
15   conformations.

Immunogenicity of refolded PvRII, ability to elicit blocking antibodies: Refolded PvRII was used to immunize rabbits and determine if it is possible to elicit inhibitory antibodies. Sera from rabbits immunized with refolded PvRII and PfF2 were tested for reactivity with refolded PvRII and PfF2, respectively, using an
20   ELISA (Figure 9). High titre rabbit antibodies that were directed against PvRII and PfF2 were detected by ELISA. The ability of these antisera to block the binding of PvRII to Duffy positive human erythrocytes was also tested. PvRII was expressed on the surface of mammalian COS cells as described earlier (Chitnis *et al*, 1994, J. Exp. Med **180**, p497-506) and tested for binding to human RBCs in the presence of
25   different dilutions of rabbit antisera raised against refolded PvRII (Table 1). Rabbit antisera completely blocked binding of RBCs to PvRII up to a dilution of 1:2500. These data indicate that refolded PvRII is immunogenic and can elicit inhibitory antibodies capable of blocking the binding of PvRII to RBCs.

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Table II. Inhibition of RBC binding to PvRII expressed on COS cell surface with rabbit antibodies raised against refolded, recombinant PvRII.

Antisera	Dilution	No. of Rosettes in 50 Fields[a]
None	NA	800
Pre-immune	1:10	850
Adjuvant control	1:10	600
Anti-PvRII	1:250	0
Anti-PvRII	1:500	0
Anti-PvRII	1:1,000	0
Anti-PvRII	1:2,500	0
Anti-PvRII	1:5,000	400
Anti-PvRII	1:7,500	450
Anti-PvRII	1:10,000	750

[a]Numbers of COS cells covered with rosettes of RBCs were scored in 50 fields at a magnification of 40. The average number of rosettes found in 3 wells is reported.

Pre-absorption of refolded PvRII with Duffy positive erythrocytes followed by detection of residual PvRII by immunoprecipitation and Western blotting was used to determine if some fraction of refolded PvRII is not functional. The sensitivity of detection of PvRII by immunoprecipitation followed by Western blotting was determined using known quantities of refolded PvRII. 10ng of refolded PvRII can be clearly detected by this method. Duffy positive and Duffy negative human erythrocytes were used to pre-absorb 8µg of refolded PvRII prior to immunoprecipitation. As expected, Duffy negative erythrocytes failed to absorb PvRII and residual PvRII was clearly detected. However, no residual PvRII was detected when Duffy positive erythrocytes were used for pre-absorption. Considering that the sensitivity of detection of PvRII was 100ng and 8µg of refolded PvRII was used, inability to detect residual protein after pre-absorption with Duffy positive erythrocytes indicates that at least 98.75% of refolded PvRII has been removed by pre-absorption and is functional (see Figure 10).

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Rabbit sera raised against refolded PfF2 and assays for inhibition of RBC binding

Antibodies against PfF2 were raised in rabbits by immunization with refolded PfF2 formulated in Freund's adjuvant. Rabbit IgG antibodies were purified and

5   tested for inhibition of erythrocyte invasion by *P. falciparum*. Late-stage schizonts of *P. falciparum* laboratory strain 3D7 and *P. falciparum* field isolate JDP8 were purified and incubated for 10 to 12 hours with human erythrocytes in the presence of different concentrations of purified rabbit antibodies raised against PfF2. Antibodies purified from rabbits immunized with adjuvant alone were used as control. Newly

10 formed rings from fresh invasions were scored by Giemsa staining and efficiency of invasion was determined. Antibodies directed against PfF2 block erythrocyte invasion efficiently with around 95% inhibition at any antibody concentration of 1 mg/ml. Importantly, antibodies directed against PfF2 block invasion of both parasite strains tested. *P. falciparum* field isolate JDP8 invades human erythrocytes using

15 multiple, sialic acid-independent pathways (Okoyeh J.N, Pillai C.R. and Chitnis C.E., 1999, *Infection and Immunity* 67, p5784-5791). These data provide evidence that antibodies raised against PfP2 will effectively block erythrocyte invasion by diverse *P. falciparum* isolates providing support for vaccine development based on PfF2.

20

Table III. Inhibition of RBC invasion with antibodies to PfF2

	Antibody concentration (mg/ml)				
P. falciparum strain	1	0.5	0.25	0.125	0.06
3D7	94.8%	92.3%	90.6%	89.0%	85.5%
JDP8	95.1%	93.5%	92.9%	88.8%	83.9%

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CLAIMS

1.   A method of producing a polypeptide comprising a Duffy binding like (DBL) domain, wherein the method comprises:

    (i)   expressing the polypeptide in a bacterium, or as a non-secreted polypeptide in a yeast;

    (ii)   extracting the expressed polypeptide from the bacterium or yeast and denaturing the polypeptide;

    (iii)   refolding the extracted polypeptide in the presence of arginine and urea; and optionally

    (iv)   recovering the refolded polypeptide.

2.   A method according to claim 1, wherein, in step (iii), multiple additions of the extracted polypeptide are made to the refolding solution.

3.   A method according to claim 1, wherein the extraction is carried out under denaturing conditions.

4.   A method according to claim 1 or 2, wherein in step (iii) the extracted protein is refolded by dilution with a solution containing the urea and arginine.

5.   A method according to claim 3 wherein the dilution is from 50 to 100 fold, preferably 100 fold.

6.   A method according to any one of the preceding claims wherein after the polypeptide has been refolded the recovery process includes removal of arginine and then subsequently removal of urea.

7.   A method according to claim 6, wherein the arginine is removed by dialysis and/or the urea is removed by ion exchange chromatography or gel filtration chromatogrpahy.

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8.   A method according to anyone of the preceding claims wherein the ratio of arginine to urea in step (iii) is from 1:1 to 1:5.

9.   A method according to claim 8 wherein the ratio is 1:2.

5

10.   A method according to any one of claims 1 to 7, wherein in step (iii) the urea is present at a concentration of from 0.5 to 1.5 M and/or the arginine is present at a concentration of from 0.1 to 1 M.

10   11.   A method according to claim 10, wherein the urea is present at a concentration of 1.0 M and the arginine is present at a concentration of 0.5 M.

12.   A method according to any one of the preceding claims, wherein step (iii) is carried out at a temperature of from 5 to 15°C and/or for a period of from 12 to 48

15   hours.

13.   A method according to claim 12, wherein step (iii) is carried out at a temperature of 10°C and/or for a period of 36 hours.

20   14.   A method according to any one of the preceding claims, wherein the bacterium is *E. coli*.

15.   A method according to anyone of the preceding claims, wherein the protein comprising a DBL domain is a *Plasmodium falciparum* or *Plasmodium vivax*,

25   polypeptide.

16.   A method according to claim 15, wherein the polypeptide is the *Plasmodium vivax* Duffy binding protein (PvDBP), the *Plasmodium falciparium* erythrocyte binding antigen (EBA-175), a PfEMP-1 family protein or a fragment variant or

30   homologue of any of these comprising a DBL domain.

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17. A method according to claim 16, wherein the polypeptide comprises region II (PvRII) of the PvDBP protein or region II (PfF2) of the EBA-175 protein or a fragment of either protein capable of specifically binding an erythrocyte.

5   18. A method according to claim 16 wherein the polypeptide has the amino acid sequence of SEQ ID NOs: 1 or 3 or a variant or fragment thereof capable of specifically binding the Duffy antigen or glycophorin.

19. A method according to anyone of the preceding claims wherein the
10   polypeptide also comprises a member of a specific binding pair to allow its recovery.

20. A method according to claim 19, wherein the member of a specific binding pair is a His tag.

15   21. A method according to any one of the preceding claims, wherein the extracted polypeptide is recovered by extracting inclusion bodies from the bacterium or yeast and recovering the polypeptide from said inclusion bodies.

22. A method according to any one of the preceding claims further comprising
20   formulating the refolded polypeptide into a pharmaceutical composition.

23. A method according to claim 22, wherein the pharmaceutical composition is a vaccine composition.

25   24. A pharmaceutical composition obtainable or obtained by the method of claim 22.

25. A vaccine composition obtainable or obtained by the method of claim 23.

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26.   A method for the identification of a substance that modulates the interaction between a polypeptide produced by a method as defined in any one of claims 1 to 21 and a host cell receptor involved in the entry of a parasite into a host cell, wherein the method comprises:

5   (i)   contacting the receptor with the polypeptide in the presence of a test substance;

           (ii)   determining the effect of the test substance on the interaction between the receptor and polypeptide thereby to determine whether the test substance is

10   capable of modulating the interaction between the receptor and polypeptide.

27.   A method according to claim 26, wherein the receptor is present on the surface of a cell.

15

28.   A method of treating or preventing malaria in an individual comprising immunising the individual with a pharmaceutical composition according to claim 22 or a vaccine composition according to claim 23.

20   29.   Use of a substance identified by a method according to claim 26 or 27 in the manufacture of a medicament for treating or preventing malaria.
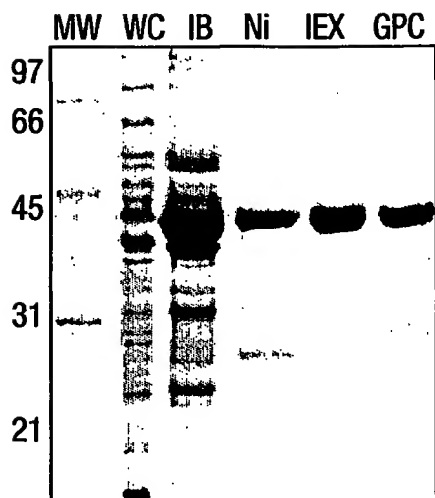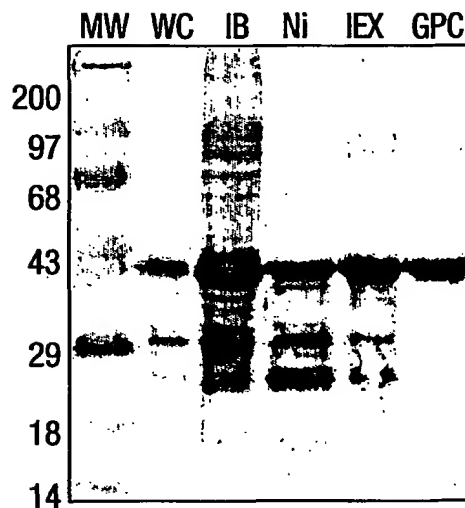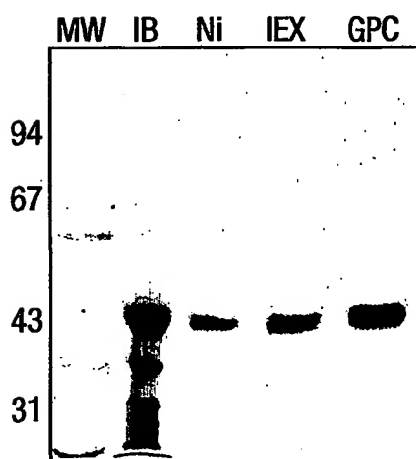
Fig.1A.

Fig.1B.

Fig.2A.

Fig.2B.
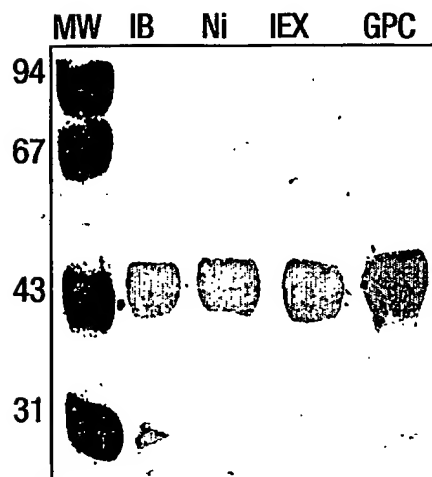
Fig.3A.

0.6   1.0   2.0   (µg)

97

66

45

31

21

Fig.3B.

0.5   1.0   2.0   (µg)

97

66

45

31

21

Fig.4A.

−DTT   +DTT

97

66

45

31

21

Fig.4B.

−DTT   +DTT

97

66

45

31

21

Fig.5A.

Fig.5B.

Fig.6A.

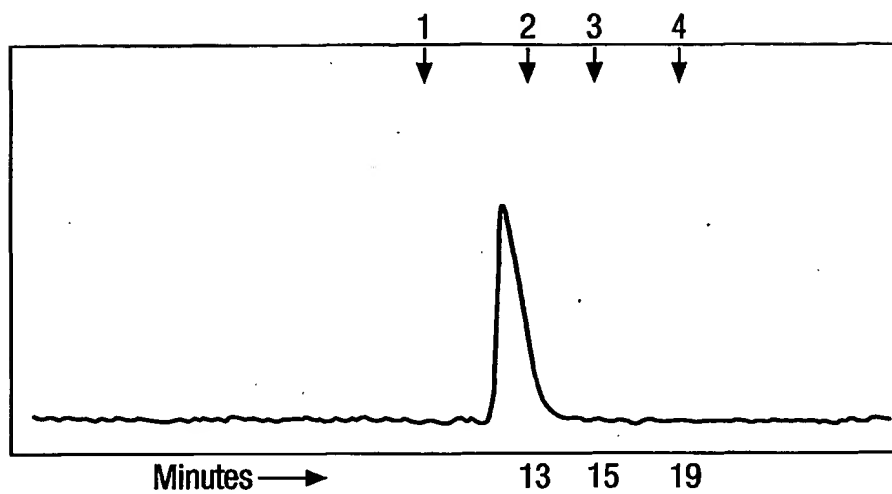# Fig.6B

Fig.7B.

Fig.7A.

Fig.8A.

RF   Fy(a+b+)   Fy(a-b-)

106
81
47
35
28

Fig.8B.

RF   Hu   Hu   Rh   Rh
             Neu   Neu

106
81
47
35
28

Fig.10.

Preabs   –   D+   D–

◄── PvRII
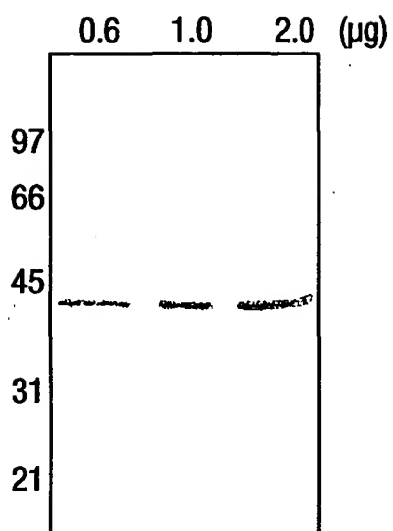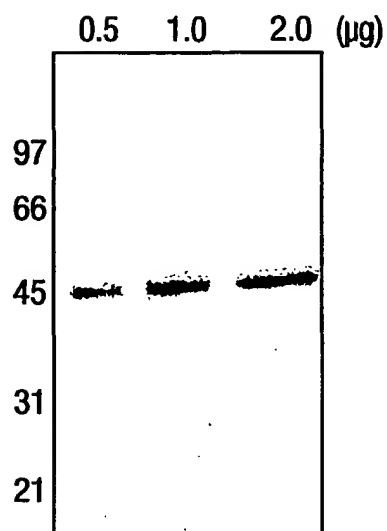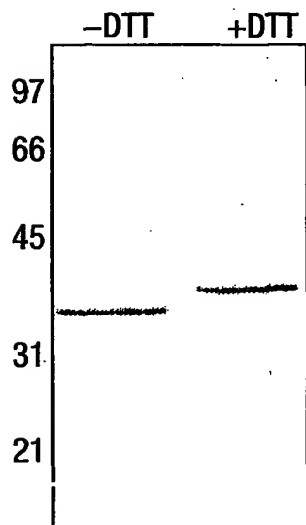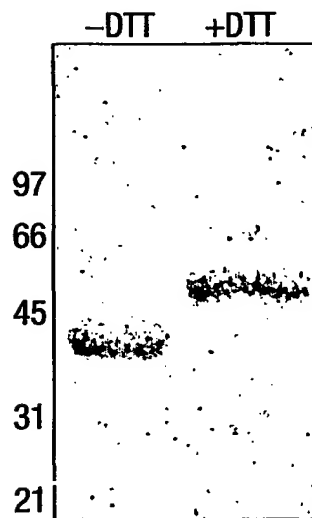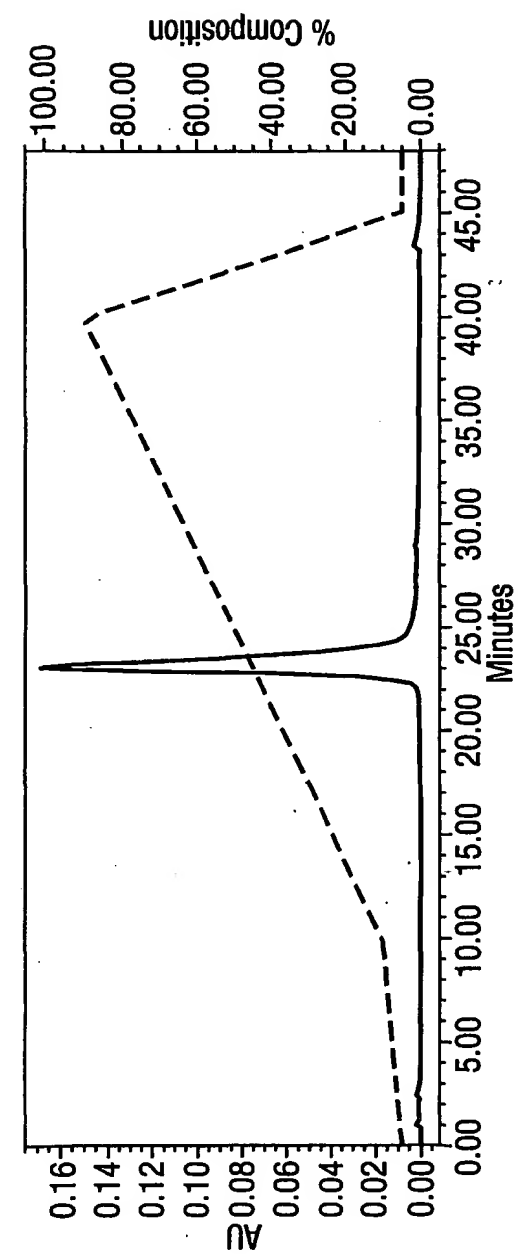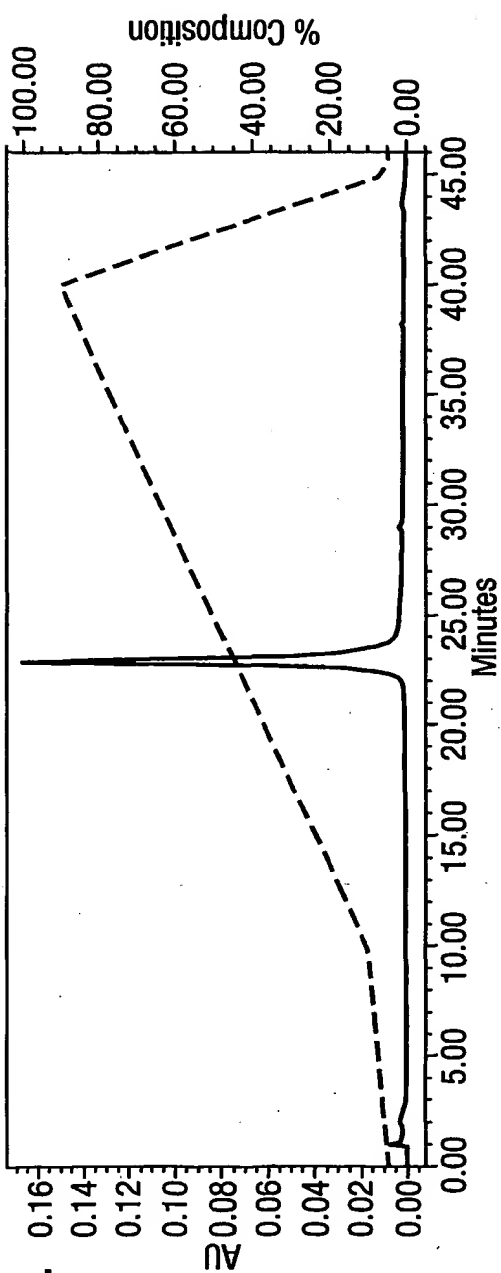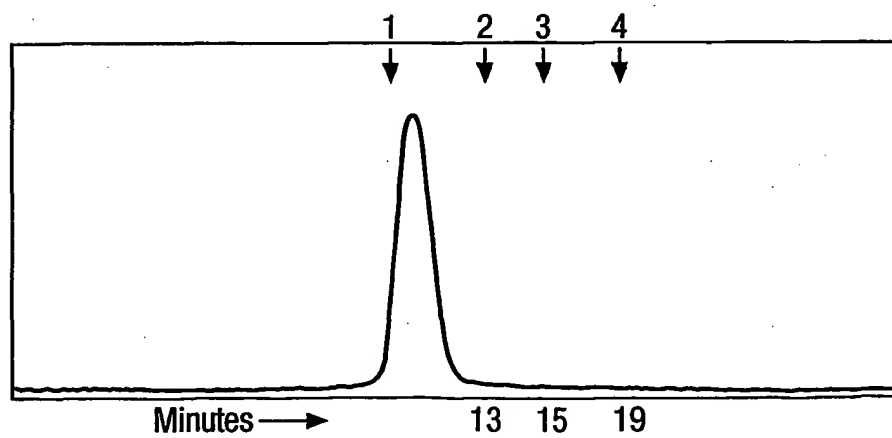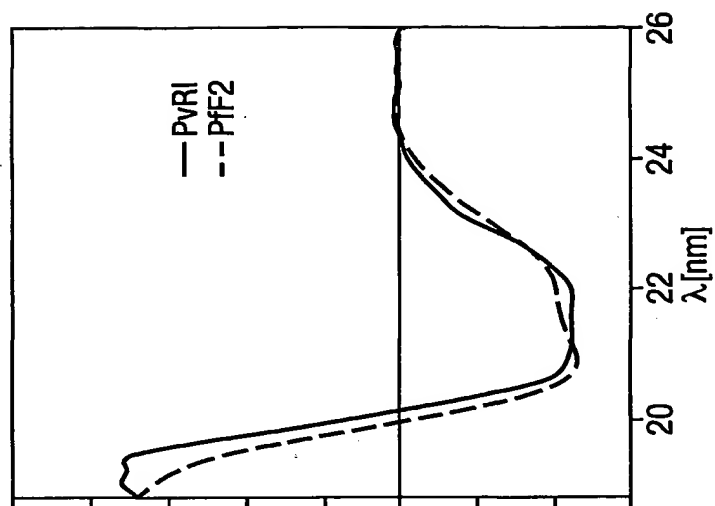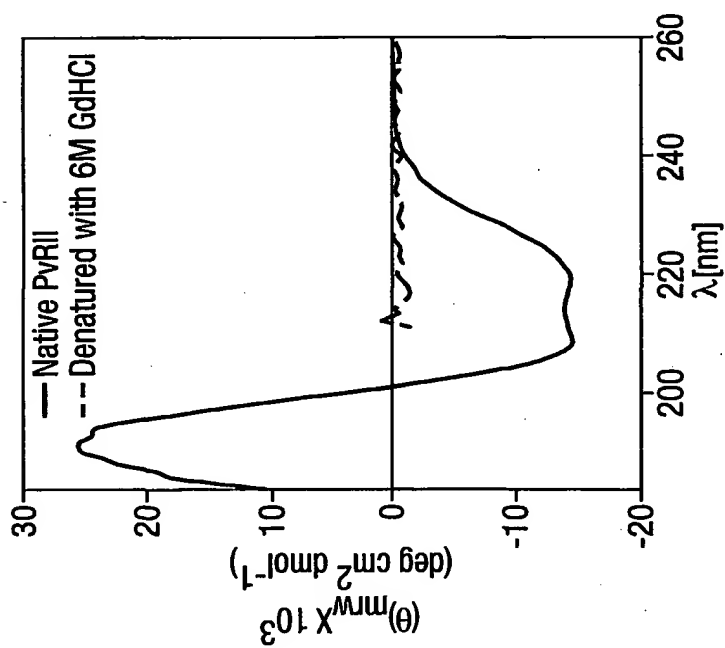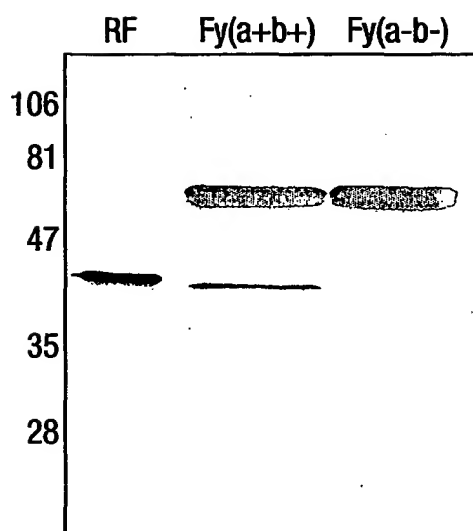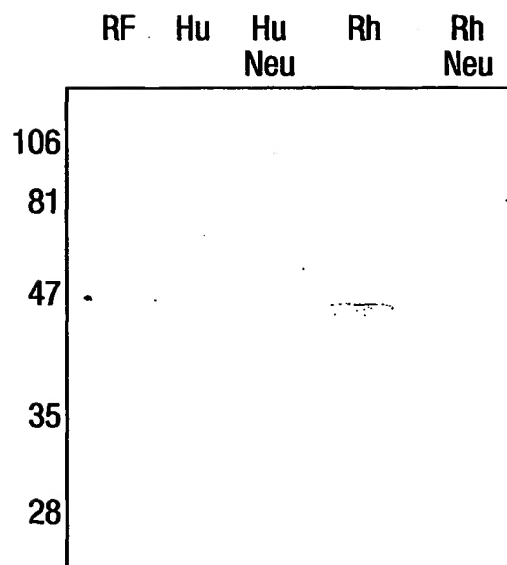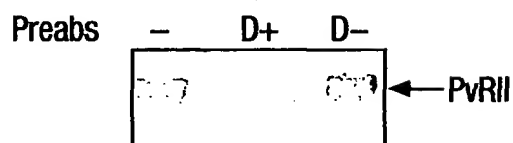
Fig.9A.

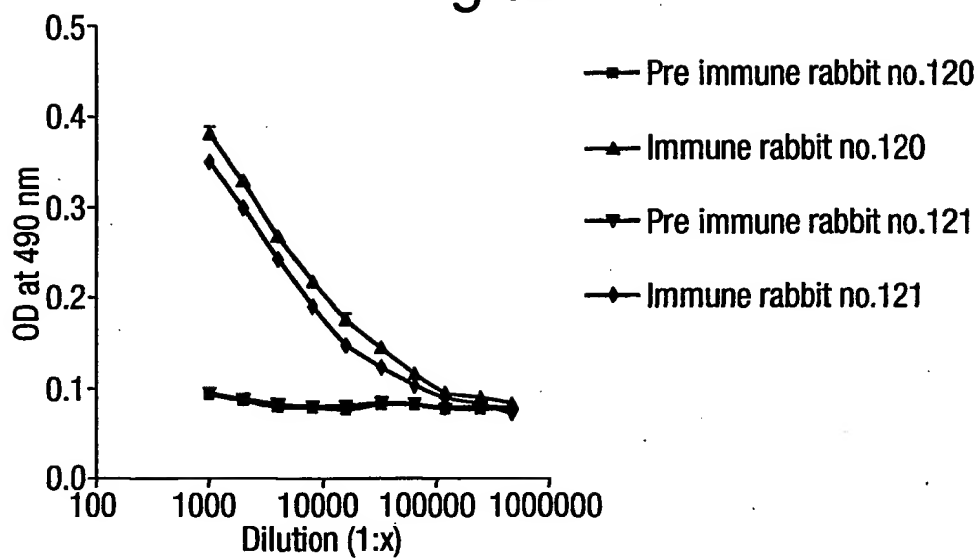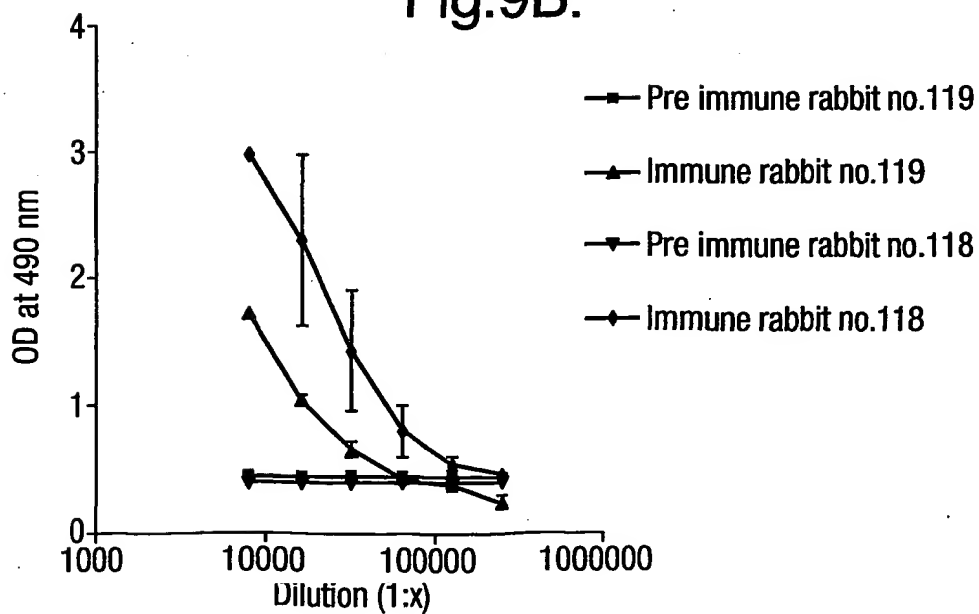## Fig.9B.

-1-

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<170> PatentIn Ver. 2.1

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Glu Arg Asp Trp Asp Cys Asn Thr Lys Lys Asp Val Cys Ile Pro Asp
        35   40   45

Arg Arg Tyr Gln Leu Cys Met Lys Glu Leu Thr Asn Leu Val Asn Asn
    50   55   60

Thr Asp Thr Asn Phe His Arg Asp Ile Thr Phe Arg Lys Leu Tyr Leu
65   70   75   80

Lys Arg Lys Leu Ile Tyr Asp Ala Ala Val Glu Gly Asp Leu Leu Leu
                85   90   95

-2-

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        Lys Leu Asn Asn Tyr Arg Tyr Asn Lys Asp Phe Cys Lys Asp Ile Arg
                    100                 105                 110
        Trp Ser Leu Gly Asp Phe Gly Asp Ile Ile Met Gly Thr Asp Met Glu
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5       Gly Ile Gly Tyr Ser Lys Val Val Glu Asn Asn Leu Arg Ser Ile Phe
                130                 135                 140
        Gly Thr Asp Glu Lys Ala Gln Gln Arg Arg Lys Gln Trp Trp Asn Glu
        145                 150                 155                 160
        Ser Lys Ala Gln Ile Trp Thr Ala Met Met Tyr Ser Val Lys Lys Arg
10                      165                 170                 175
        Leu Lys Gly Asn Phe Ile Trp Ile Cys Lys Leu Asn Val Ala Val Asn
                    180                 185                 190
        Ile Glu Pro Gln Ile Tyr Arg Trp Ile Arg Glu Trp Gly Arg Asp Tyr
                    195                 200                 205
15      Val Ser Glu Leu Pro Thr Glu Val Gln Lys Leu Lys Glu Lys Cys Asp
                210                 215                 220
        Gly Lys Ile Asn Tyr Thr Asp Lys Lys Val Cys Lys Val Pro Pro Cys
        225                 230                 235                 240
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20                      245                 250                 255
        Gln Trp Asp Val Leu Ser Asn Lys Phe Ile Ser Val Lys Asn Ala Glu
                    260                 265                 270
        Lys Val Gln Thr Ala Gly Ile Val Thr Pro Tyr Asp Ile Leu Lys Gln
                    275                 280                 285
25      Glu Leu Asp Glu Phe Asn Glu Val Ala Phe Glu Asn Glu Ile Asn Lys
                290                 295                 300
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         35   40   45

Leu Ser Ile Ile Lys Thr Tyr Thr Lys Glu Thr Met Lys Asp His Phe
     50   55   60

Ile Glu Ala Ser Lys Lys Glu Ser Gln Leu Leu Leu Lys Lys Asn Asp
 65   70   75   80

Asn Lys Tyr Asn Ser Lys Phe Cys Asn Asp Leu Lys Asn Ser Phe Leu
                 85   90   95

Asp Tyr Gly His Leu Ala Met Gly Asn Asp Met Asp Phe Gly Gly Tyr
            100   105   110

Ser Thr Lys Ala Glu Asn Lys Ile Gln Glu Val Phe Lys Gly Ala His
        115   120   125

Gly Glu Ile Ser Glu His Lys Ile Lys Asn Phe Arg Lys Glu Trp Trp
        130   135   140

Asn Glu Phe Arg Glu Lys Leu Trp Glu Ala Met Leu Ser Glu His Lys
145   150   155   160

Asn Asn Ile Asn Asn Cys Lys Asn Ile Pro Gln Glu Glu Leu Gln Ile
                165   170   175

Thr Gln Trp Ile Lys Glu Trp His Gly Glu Phe Leu Leu Glu Arg Asp
            180   185   190

Asn Arg Ser Lys Leu Pro Lys Ser Lys Cys Lys Asn Asn Thr Leu Tyr
        195   200   205

Glu Ala Cys Glu Lys Glu Cys Ile Asp Pro Cys Met Lys Tyr Arg Asp
    210   215   220

Trp Ile Ile Arg Ser Lys Phe Glu Trp His Thr Leu Ser Lys Glu Tyr
225   230   235   240

Glu Thr Gln Lys Val Pro Lys Glu Asn Ala Glu Asn Tyr Leu Ile Lys

-4-

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               245                250                255
     Ile Ser Glu Asn Lys Asn Asp Ala Lys Val Ser Leu Leu Asn Asn
               260                265                270
     Cys Asp Ala Glu Tyr Ser Lys Tyr Cys Asp Cys Lys His Thr Thr Thr
5              275                280                285
     Leu Val Lys Ser Val Leu Asn Gly Asn Asp Asn Thr Ile Lys
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20   Asn Pro Tyr Ile Leu Ser Thr Lys Asp Val Cys Val Pro Pro Arg Arg
               35             40                45
     Gln Glu Leu Cys Leu Gly Asn Ile Asp Arg Ile Tyr Asp Lys Asn Leu
               50             55             60
     Leu Met Ile Lys Glu His Ile Leu Ala Ile Ala Ile Tyr Glu Ser Arg
25   65             70             75             80
     Ile Leu Lys Arg Lys Tyr Lys Asn Lys Asp Asp Lys Glu Val Cys Lys
               85             90             95
     Ile Ile Asn Lys Thr Phe Ala Asp Ile Arg Asp Ile Ile Gly Gly Thr
               100            105            110
30   Asp Tyr Trp Asn Asp Leu Ser Asn Arg Lys Leu Val Gly Lys Ile Asn
               115            120            125
     Thr Asn Ser Lys Tyr Val His Arg Asn Lys Lys Asn Asp Lys Leu Phe
               130            135            140
     Arg Asp Glu Trp Trp Lys Val Ile Lys Lys Asp Val Trp Asn Val Ile
35   145            150            155            160
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-5-

Ser Trp Val Phe Lys Asp Lys Thr Val Cys Lys Glu Asp Asp Ile Glu
                165   170   175

Asn Ile Pro Gln Phe Phe Arg Trp Phe Ser Glu Trp Gly Asp Asp Tyr
                180   185   190

5   Cys Gln Asp Lys Thr Lys Met Ile Glu Thr Leu Lys Val Glu Cys Lys
                195   200   205

Glu Lys Pro Cys Glu Asp Asp Asn Cys Lys Ser Lys Cys Asn Ser Tyr
                210   215   220

10   Lys Glu Trp Ile Ser Lys Lys Lys Glu Glu Tyr Asn Lys Gln Ala Lys
      225   230   235   240

Gln Tyr Gln Glu Tyr Gln Lys Gly Asn Asn Tyr Lys Met Tyr Ser Glu
                245   250   255

Phe Lys Ser Ile Lys Pro Glu Val Tyr Leu Lys Lys Tyr Ser Glu Lys
15   260   265   270

Cys Ser Asn Leu Asn Phe Glu Asp Glu Phe Lys Glu Glu Leu His Ser
                275   280   285

Asp Tyr Lys Asn Lys Cys Thr Met Cys Pro Glu Val Lys Asp Val Pro
                290   295   300

20   Ile Ser Ile Ile Arg Asn Asn Glu Gln Thr Ser Gln Glu Ala Val Pro
      305   310   315   320

Glu Glu Asn Thr Glu Ile Ala His Arg Thr Glu Thr Pro Ser Ile Ser
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